

Specific IgE Levels to *Cicer arietinum* (Chick Pea) in Tolerant and Nontolerant Children: Evaluation of Boiled and Raw Extracts

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Key Words

Food hypersensitivity · Legumes · Specific IgE · Chick pea · Allergens · *Cicer arietinum*

Abstract

The chick pea, *Cicer arietinum*, is a legume commonly consumed in Spain and other Mediterranean countries. The sera of 29 children (mean age: 8.4 years) with a current or past history of allergic reactions after ingestion of chick pea, and positive skin tests to this legume, were used to study the allergenic composition of raw and boiled chick pea extracts. The patient population was divided into 2 groups: group 1 consisted of 19 patients with clinical sensitivity confirmed by either positive oral challenges or a convincing recent history of anaphylaxis after eating chick peas, and group 2 consisted of 10 patients with clinical sensitivity in the past, but tolerant at the time of blood extraction. Six atopic children, not allergic to legumes, were included as controls. Specific IgE to the raw and boiled extracts was measured by ELISA. The allergenic composition of both extracts was analyzed by SDS-PAGE and immunoblots. There were

no significant differences between specific IgE levels to the raw and boiled extracts ($p = 0.23$). The mean levels in group 1 were significantly higher than in group 2 and controls ($p = 0.0001$). Multiple IgE binding proteins/peptides were detected in both extracts in the molecular weight range of 10–106 kD. Only nontolerant patients recognized a similar number of bands in both extracts. Chick pea extracts contain a majority of heat-stable allergens, which could be responsible for the clinical sensitivity to chick pea. Patients with a current clinical allergy to chick pea have statistically higher specific IgE levels than tolerant patients and controls.

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Introduction

Legumes are an important component of the Mediterranean diet. In Spain, the intake of lentils, chick peas and white beans is very common. Sensitization to these legumes is relatively frequent, mainly in the pediatric population. Among Spanish children under 5 years of age, it is the fifth most prevalent food allergy [1]. Although some

legume allergens have been identified and characterized [2–5], studies investigating the allergenicity of chick peas are scarce. So far, allergens of chick pea have not been purified or immunochemically characterized. Recently Niphadkar et al. [6] described a case of chick pea-induced anaphylaxis and detected by immunoblotting multiple IgE binding bands with molecular weights between 10 and 70 kD.

Many food allergens are proteins resistant to heat. The heating process causes the loss of conformational IgE-binding epitopes, but the resistance of some food allergens to heat denaturation suggests that conformational epitopes are not always critical for IgE binding [7]. Little information is currently available about the heat resistance of legume allergens [8, 9].

This study describes the allergenicity of raw and boiled chick pea extracts using sera of children who currently are, or were clinically sensitive to this legume. The objectives were: (1) to measure specific IgE levels to chick pea in children with clinical sensitivity to this legume, in children who were tolerant at the time of inclusion in the study and in control atopic children; (2) to evaluate the effect of heating on the *in vitro* allergenicity of chick pea extracts, and (3) to identify the allergenic composition of chick peas by immunoblots.

Materials and Methods

Patient Population

Twenty-nine chick-pea-sensitive patients were enrolled in this cross-sectional study; 23 were male (79%) and 6 female (21%), with an average age of 8.4 years (range 3–20 years). All patients had consulted a physician during the previous 10 years because of an allergic reaction after ingesting chick peas. The patients were divided into 2 groups. Group 1 consisted of 19 clinically sensitive children (mean age 8.2 years), as confirmed by either positive open food challenges, or a convincing recent history of allergic reaction. Briefly, the oral food challenge started with approximately half the dose reported to produce symptoms and was doubled every 30 min until the patient presented convincing symptoms; all patients had a positive skin test to a heated commercial, nonstandardized chick pea extract (C.B.F. Leti, Madrid, Spain; 10 mg/ml). Group 2 consisted of 10 children (mean age 9.5 years) who were tolerant at the time of entering the study; 9 had a positive skin test to the same chick pea extract. As a control group, sera from 6 atopic children (4 sensitive to pollen and 2 to mites) with no history of intolerance or food allergy and negative skin prick tests to legumes were used. After informed consent was obtained, 5 ml of blood was drawn from each child and the resulting serum was distributed in aliquots and kept at –20 °C.

Allergen Extracts

One hundred grams of chick peas were ground and extracted in 2 liters of a 0.85% NaCl solution in a shaker for 16 h at 4 °C. Half of

this extract was boiled for 15 min. Afterwards, raw and boiled extracts were centrifuged at 10,000 rpm for 30 min. After sterile filtration, both extracts were lyophilized separately.

Determination of the Protein Content

The protein concentration in both chick pea extracts was estimated by the Biuret-Lowry method (Sigma Diagnostics, St. Louis, Mo., USA), following the manufacturer's instructions. The protein content of the raw extract was 2.58 mg/ml, and of the boiled extract 1.79 mg/ml.

Specific IgE by Direct ELISA and Specific IgE Inhibitions

Specific IgE binding to the raw and boiled chick pea extracts and inhibition experiments were conducted using methods previously described [10, 11]. A result was considered positive when a serum bound 4 times more than a negative control. Briefly, 100- μ l aliquots of chick pea extracts (crude or boiled), at a protein concentration of 20 μ g/ml in sodium bicarbonate buffer (0.2 M, pH 9.2), were coated to the surface of plastic microtiter plates (Immulon 4, Chantilly, USA) and, after overnight incubation in a humid chamber, were blocked with 1% HSA in PBS for 1 h at room temperature (RT). After 3 washings, 100 μ l of the individual serum samples, in duplicate, at a dilution of 1:2, were incubated overnight. After this incubation period and further washings, 100 μ l of anti-human IgE (Ingenasa, Madrid, Spain) labeled with horseradish peroxidase (1 μ g/ml in blocking solution) were added and left to stand for 2 h at RT. After 5 washings, the horseradish peroxidase was evaluated by incubation with a development solution containing 0.16 M acetate buffer (pH 6; 1 ml), tetramethylbenzidine (TMB; Boehringer Mannheim, Mannheim, Germany) in DMSO (Merck, Darmstadt, Germany; 20 μ l) and 1 M H₂O₂ (2 μ l). The enzymatic reaction was stopped by the addition of 100 μ l 2 N sulfuric acid. Optical densities were read at 450 nm in a microtiter plate reader (Titertek Multiskan, Finland).

Specific IgE inhibition experiments were conducted in a similar manner; 125 μ l of serial dilutions of crude or boiled chick pea extracts (at equal protein concentrations), respectively, were mixed with 125 μ l of a serum pool, diluted 1:10, containing high titers of specific IgE to chick pea allergens. These mixtures were shaken for 2 h at RT. After the inhibition period, 100 μ l of these mixtures were added to the coated wells (as previously described) for an overnight incubation. Afterwards, the assay was completed as for the specific IgE determinations.

SDS-PAGE and Immunoblots

Separation by electrophoresis was accomplished in acrylamide gels according to the method of Laemmli [12]. Raw and boiled extracts were used under reduced conditions; 15% separating and 4% packing gels were used. The samples were dissolved in a Tris-HCl (pH 6.8) tampon containing 10% glycerol, 2% SDS and 5% mercaptoethanol. Before loading the gel, the samples were heated at 95 °C for 10 min. The gel was run in a Tris-HCl 62.5 mM tampon with 0.1% SDS. As molecular markers, we used the commercial standards of low range molecular size by Bio-Rad (Richmond, Va., USA). The electrical field conditions were 180 V, 35 mA and 8 W and were set in a Bio-Rad unit connected to a cooling bath. The gel was stained with 0.1% Coomassie blue (R-250) and destained in water:methanol:acetic acid (50:40:10) solution.

Immunoblots with individual sera were carried out with the raw extract and with pools to both extracts. Gel proteins were transferred to Immobilon membranes (Millipore, Bedford, Mass., USA) in a

Table 1. Clinical features and laboratory findings in groups 1 and 2 patients

Features	Group 1 (n = 19)	Group 2 (n = 10)
History of atopy ^a	16/19 (84)	10/10 (100)
Mean age at first reaction, years	2.1 (0.5–8)	4.1 (1–12)
Mean age at tolerance, years	–	9.5 (3–20)
Symptoms ^b		
Skin	9/14 (64)	7/10 (70)
Respiratory	8/14 (57)	3/10 (30)
Gastrointestinal	2/14 (14.3)	2/10 (20)
Clinical allergy to other legumes		
Lentils	16/19 (84)	5/10 (50)
Peas	13/19 (68)	1/10 (10)
Peanuts	2/19 (10)	1/10 (10)
Positive skin prick tests to other legumes		
Lentils	19/19 (100)	8/10 (80)
Peas	18/19 (95)	4/10 (40)
Peanuts	13/19 (68)	4/10 (40)
Mean total IgE, kU/l	473 (25–1,878)	685 (104–1,868)

^a Described as the concomitant presence of other positive skin tests and allergic diseases.

^b As confirmed by oral challenges; 5 patients of group 1 were not challenged because they had a convincing recent history of anaphylaxis to chick pea (2 systemic anaphylaxis; 2, asthma after exposure to steam from cooked chick peas; 1 several episodes of generalized urticaria). All patients of group 2 were challenged; results at the time of the study were negative. Symptoms: skin: urticaria and/or angioedema; respiratory: cough, wheezing, rhinitis, conjunctivitis, and/or laryngeal edema; gastrointestinal: vomiting.

Figures are numbers and percentage (in parentheses) where not indicated otherwise.

Trans-Blot (Bio-Rad) tank in a basic buffer. After the transfer, the membranes were allowed to dry and incubated overnight in a humid chamber at RT with the individual sera (diluted 1:3), or serum pools of groups 1, 2 and control patients (diluted 1:40) in a PBS 0.1%, Tween 20 solution. The strips were then washed with 0.1% PBS Tween and incubated for 2 h at RT with peroxidase-labeled monoclonal human anti-IgE (anti-IgE-PO; Ingenasa) at a concentration of 1 µg/ml and 3% HSA. After the corresponding washes, the enzymatic reaction took place in a solution composed of a 0.1 M citrate tampon (pH 5.0) containing 10 mM EDTA and 1% dextran sulfate (1 ml), TMB in DMSO (20 µl) and 1 M H₂O₂ (2 µl).

Results

The clinical features of group 1 and group 2 patients are shown in table 1. The mean age at the time of the first reaction was lower in group 1 than in group 2. In group 1,

Table 2. ELISA results (OD) in group 1, group 2 and control group patients

	Raw extract	Boiled extract
Group 1 (n = 19)	1.97 3.13 2.12 3.15 2.99 3.03 2.91 3.19 3.31 0.77 2.88 0.26 2.43 2.10 1.91 3.32 3.07 1.27 2.81	1.51 3.38 1.80 3.55 3.03 3.38 2.94 3.17 3.35 0.92 2.53 0.28 1.24 1.71 1.70 3.20 3.14 2.28 2.81
Group 2 (n = 10)	0.61 0.88 0.09 2.00 0.53 2.45 0.31 0.92 0.42 0.04	0.70 0.71 0.10 1.21 0.24 1.89 0.17 0.25 0.48 0.05
Control group (n = 6)	0.10 0.06 0.06 0.08 0.02 0.06	0.07 0.05 0.05 0.04 0.02 0.07

Specific IgE levels in group 1 were significantly higher than in group 2 for both extracts (p = 0.0001). Specific IgE in the control group was negative using both extracts.

the clinical sensitization to other legumes, confirmed by oral challenges, was more frequent than in group 2 especially to lentils and peas. Other characteristics were similar in both groups.

Specific IgE levels in group 1 were significantly higher than in group 2 for both extracts (p = 0.0001). Table 2 shows the individual ELISA results. The effect of boiling on specific IgE binding to chick pea did not show statisti-

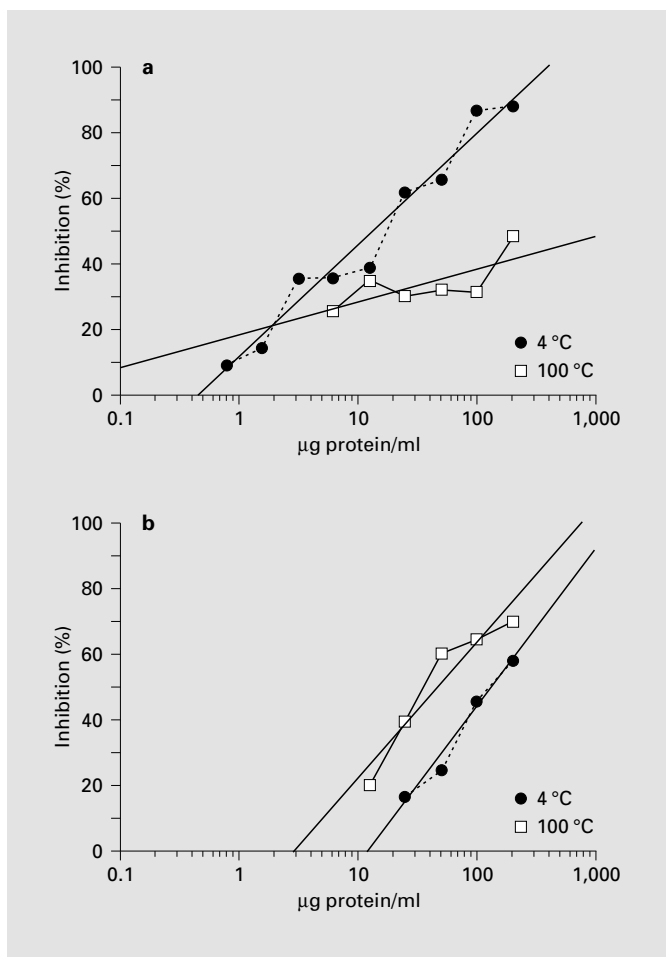


Fig. 1. ELISA inhibition curves using the raw (a) and boiled (b) chick pea extracts in the solid phase.

cally significant differences (Wilcoxon signed-rank test) for either group (group 1: $p = 0.903$; group 2: $p = 0.074$). Specific IgE in control group was negative using both extracts.

Specific IgE inhibition experiments were conducted using both extracts at the same protein concentrations in the solid and fluid phases (fig. 1). Different slopes were observed when the raw extract was used in the solid phase (fig. 1a). In this system, the raw extract showed a greater inhibitory capacity than the boiled extract. However, when the boiled extract was used in the solid phase (fig. 1b), the slopes of the curves were similar and the boiled extract showed a greater inhibitory capacity in its homologous system.

Figure 2 shows the protein distribution of the raw and boiled chick pea extracts under reduced conditions. Some

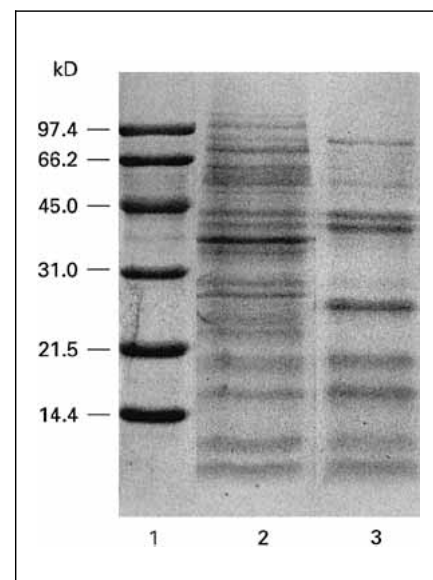


Fig. 2. SDS-PAGE of raw and boiled chick pea extracts after reduction with mercaptoethanol. Lane 1: molecular weight standard; lane 2: raw chick pea extract; lane 3: boiled chick pea extract.

differences were observed between the SDS-PAGE patterns of the raw and boiled extracts; protein bands with molecular weights below 21.5 kD were similar in both extracts. The disappearance of certain bands was observed at higher molecular weights, especially above 31 kD.

Immunoblots performed with the serum pool from group 1 patients identified multiple protein bands in the molecular weight range of 10–106 kD. Overall, more intense staining was observed with the raw extract. However, two bands between 60 and 80 kD retained a similarly intense specific IgE binding. The serum pool of group 2 patients detected fewer bands in the boiled than in the raw extract. The immunoblot performed with the serum pool of control individuals, with a negative specific IgE determination, revealed binding to some protein bands in the raw but not in the boiled extract (fig. 3).

Immunoblots with the individual sera of group 1 patients were performed with the raw extract. More than 80% of the sera detected protein bands below 20 kD. Other bands of greater molecular weight were also detected by over 50% of the sera (21.8, 27.7, 36.1, 50.9, 51.8, and 69.8) (fig. 4).

Fig. 3. SDS-PAGE immunoblots of raw and boiled chick pea extracts under reduced conditions using a serum pool of group 1 (lanes 1 and 2), a serum pool of group 2 (lanes 3 and 4) and a serum pool of the control group (lanes 5 and 6).

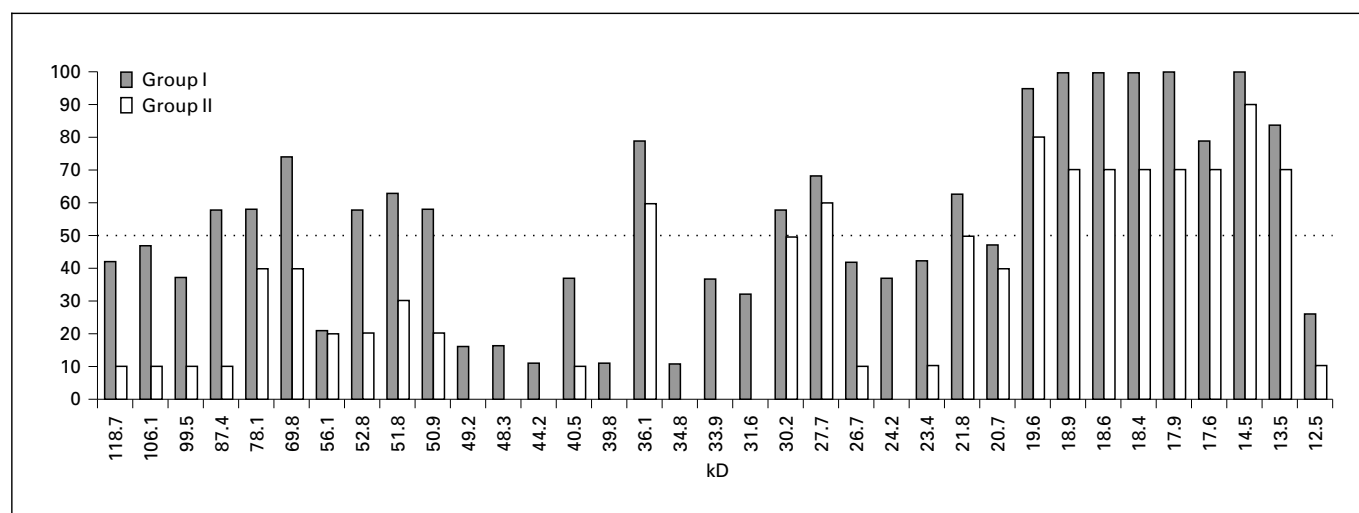
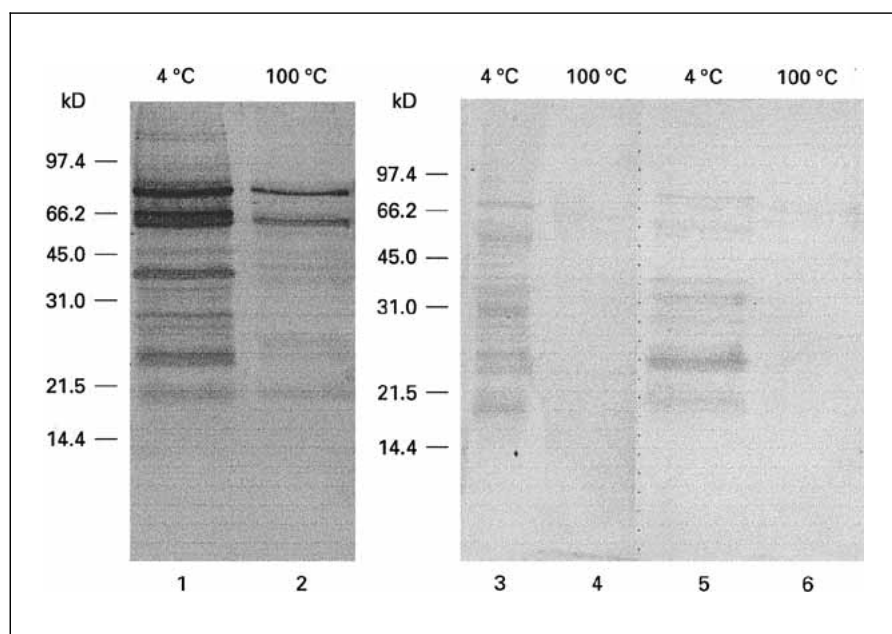


Fig. 4. Frequency of allergen recognition by individual sera in group 1 and group 2 patients. More than 80% of the sera in group 1 recognized allergens below 20 kD. Other bands of greater molecular weight were also detected by more than 50% of the sera (21.8, 27.7, 36.1, 50.9, 51.8, 69.8, 78.1, 87.4).

Discussion

This study is the first attempt to characterize the allergenicity of raw and boiled chick pea extracts and describes lower specific IgE levels to both chick pea extracts in a group of children who became tolerant to the ingestion of this legume. We acknowledge, however, that spe-

cific IgE levels in this group were not tested prior to entry into the study. These data are needed to evaluate the role of specific IgE in food allergy in a more conclusive way. Group 1 children are now being followed to identify a reduction in specific IgE levels with a concomitant appearance of tolerance.

There is little published information about the natural history of legume allergy, with the exception of the peanut. A retrospective study by García-Ara et al. [13] found that 18% of 17 children allergic to legumes and nuts became tolerant after a mean follow-up period of 6 years. These authors also detected lower specific IgE levels in the group of tolerant children compared to the clinically sensitive group and identified a reduction in specific IgE levels when the patients became tolerant.

The results of our study suggest that the appearance of tolerance to chick peas may not only be age-dependent but also related to diminishing specific IgE levels, probably due to a dietary avoidance. Other factors, such as earlier contact with the food, age at the onset of symptoms, greater sensitivity to heat-stable allergens and genetic markers, may also be contemplated.

The effect of temperature on legume allergens has scarcely been studied. Burks et al. [8] did not observe any decrease in the allergenicity of the peanut extract after heating, and Carrillo et al. [9] also suggested that some lentil allergens may be thermostable. In our study, specific IgE levels to the raw and heated extracts were similar, which indicates the existence of a majority of thermostable allergens in chick pea extracts. Specific IgE inhibition experiments demonstrated that both extracts exhibited a greater inhibitory capacity when used in their respective homologous systems. However, parallel lines were only obtained when the boiled extract was used in the solid phase. These data suggest that IgE antibodies may have more affinity for heated allergens, since chick peas for human consumption are always roasted or boiled. The contribution of thermostable allergens to the total allergenicity of chick peas seems to be very important. The differences of the slopes seen in both solid phases also indicate the denaturation of some allergens. Heating may be conducive to the formation of neo-antigens or to the breakdown of proteins in smaller allergenic peptides, as has been shown in other studies [14, 15]. Chick pea allergens could be pH-resistant and resist the passage through the stomach. This is suggested by a case of chick-pea-induced-anaphylaxis which occurred 1 h after the ingestion of this food [6].

In our immunoblot studies, we have observed that only the serum pool of children with clinical sensitivity recognized a similar number of protein bands in both extracts (raw and boiled). However, the disappearance of certain bands could have been a result of the filtration step following cooking. Children who had developed tolerance to chick peas detected protein bands almost exclusively in the raw extract. Nonspecific binding to raw extract was

detected by the serum pool of the control group. This non-specific binding to legume allergens has been reported by other authors [16, 17], and it was less intense than in group 1 patients and took place in spite of a negative specific IgE determination and negative skin tests.

Immunoblot showed the detection of numerous IgE-binding bands. These data are in agreement with previous studies. Niphadkar et al. [6] detected multiple IgE-binding bands in a chick pea extract. Bernhisel-Broadbent et al. [17] identified numerous bands in a chick pea extract using sera of peanut-allergic patients. Two important peanut allergens have molecular weights of 63.5 kD (Ara h 1) and 17 kD (Ara h 2) [2, 3], and 2 protein bands with similar molecular weights were also detected by IgE of group 1 patients in our study (62.4 and 18.6 kD, respectively). These data suggest the existence of a certain degree of cross-reactivity between these legumes, at least in vitro, as has been demonstrated in other studies [17, 18].

The results of our study contribute to the knowledge of the allergenicity of the chick pea and suggest that the main allergens are heat-stable proteins, as showed by ELISA, immunoblot experiments and skin testing. These heat-resistant allergens, or neo-allergens, may be relevant to the clinical manifestations of chick pea sensitivity. The appearance of tolerance to chick peas seems to be associated with a reduction in specific IgE levels to chick pea allergens.

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